Kidney International, Vol. 65 (2004), pp. 1856–1863

Nephrin gene (*NPHS1*) in patients with minimal change nephrotic syndrome (MCNS)

Anne-Tiina Lahdenkari, Marjo Kestilä, Christer Holmberg, Olli Koskimies, and Hannu Jalanko

Hospital for Children and Adolescents and Biomedicum Helsinki, University of Helsinki, Helsinki, Finland; and Department of Molecular Medicine, National Public Health Institute, Helsinki, Finland

Nephrin gene (*NPHS1*) in patients with minimal change nephrotic syndrome (MCNS).

Background. Minimal change nephrotic syndrome (MCNS) is a major problem in pediatric nephrology. While the pathogenesis of MCNS is not known, the latest discoveries in the genetic diseases indicate that glomerular epithelial cells (podocytes) and the slit diaphragm play a primary role in development of proteinuria. Because nephrin is known to be a major component of the slit diaphragm, we analyzed the structure of nephrin gene (*NPHS1*) in patients with MCNS of different severity.

Methods. Clinical data and DNA samples were collected from 25 adults who had biopsy-proven MCNS in childhood. A direct sequencing was performed to all 29 exons of the NPHS1 gene. The significance of the findings was evaluated by similar analysis of DNA samples from 25 healthy control patients.

Results. The analysis of *NPHS1* revealed no specific MCNS-associated mutation. However, 5 of the 25 MCNS patients had heterozygous allelic variants leading to nonconservative amino acid substitutions not previously reported (G879R; R800C; T294I; A916S). One of the five patients also had the Fin-major mutation, and two had new, conservative amino acid substitutions (S786N; A342G). Three of the five patients were classified as steroid sensitive, one was an early nonresponder, and one patient showed clear resistance to steroid treatment. Six known polymorphic changes in *NPHS1* were also found, three of them leading to amino acid changes. The number of allelic variants was high both in MCNS patients and control patients (mean 3.0 and 2.6).

Conclusion. The results suggest that genetic changes in nephrin may have a pathogenetic role in some patients with MCNS.

Minimal change nephrotic syndrome (MCNS) is the most common form of nephrotic syndrome in childhood. While most patients respond to steroid treatment, the disease is characterized by more or less frequent relapses [1, 2]. The pathophysiology of MCNS is not known. Glucocorticoids and other immunomodulating drugs induce remission, suggesting that MCNS has an immunologic basis. No visible inflammation is present in kidney glomerulus, and it has been postulated that T lymphocytes might produce a circulating factor that alters the glomerular permeability of the glomerular capillary wall [3]. However, no such factor has been isolated.

It is also unknown which part of the glomerular sieve is affected in MCNS. The glomerular filter is composed of three layers: a fenestrated endothelium, the glomerular basement membrane, and podocyte foot processes connected by the slit diaphragm. Recent data on genetic diseases clearly indicate that the slit diaphragm is crucial in preventing the leakage of the plasma proteins into urine [4–6]. Nephrin is a major component of the slit diaphragm. It is a transmembrane protein belonging to the immunoglobulin superfamily containing eight extracellular immunoglobulin (Ig) domains and one fibronectin motif (Fig. 1). Mutations in the nephrin gene (*NPHS1*) are responsible for the congenital nephrotic syndrome of the Finnish type (CNF, NPHS1), which is characterized by heavy proteinuria starting already in utero [7, 8]. The disease is highly enriched in Finland, with a frequency of 1/8200 newborns [9]. *NPHS1* consists of 29 exons and has a size of 26 kb. The Finnish CNF children have two mutations, Fin-major in exon 2 and Fin-minor in exon 26, which account for 97% of the cases in Finland [7]. Both mutations lead to a truncated protein, which is not expressed on the podocyte surface [10]. In contrast to this, the non-Finnish patients have "individual" mutations, and more than 60 mutations along *NPHS1* have so far been described [11–13]. In the Finnish CNF patients, lack of nephrin in the kidney glomerulus leads to absence of the slit diaphragm, as studied by electron microscopy [10].

What the role of nephrin and the slit diaphragm is in MCNS is not known. We recently found that the slit diaphragm is often missing in MCNS kidneys, similar to

Key words: nephrin, NPHS1, minimal change nephrosis, slit diaphragm.

Received for publication March 19, 2003
and in revised form June 19, 2003, September 3, 2003, and November 10, 2003
Accepted for publication December 9, 2003

Table 1. Characteristics of the MCNS patients

Feature	No.
No. of patients	25
Male/female	19/6
Age at onset *years*	4.9 (0.8–14.5)[a]
Duration of disease *years*	6.6 (0.3–27)[a]
Number of relapses	9.4 (0–28)[a]
Steroid sensitive/resistant	20/5[b]
Infrequent/frequent relapses	12/13[c]
Steroid dependence: no/yes	19/6
Follow-up data	
Age *years*	35.2 (25.8–42.4)[a]
Proteinuric episodes continue: no/yes	22/3
Renal failure: no/yes	25/0

[a]Mean (range).
[b]Steroid resistance refers to cases with proteinuric episodes not responding to prednisolone therapy in four weeks.
[c]Frequent relapses refer to cases with at least four episodes/year.

that in CNF [14]. Also, altered expression of nephrin has been reported in MCNS kidneys [15, 16]. Whether these are primary or secondary changes is not clarified. Nephrin synthesis in MCNS kidneys has been reported to be normal in most published studies [17, 18]. However, one can speculate that qualitative, genetic changes in nephrin might make the protein functionally or structurally defective, and such a "weak" component would render the glomerular filter vulnerable to pathogenic processes (e.g., an immunologic attack.

This prompted us to study the structure of nephrin gene in patients with MCNS. The Finnish patients were especially suitable for this analysis because the Finns are genetically homogenous and have a high frequency of the two *NPHS1* mutations. Because of this founder effect one might expect to find only a limited number of possible nucleotide changes predisposing to MCNS. For the genetic analysis we chose 25 adults who had been treated for MCNS in childhood, so that any mistake in the diagnosis or outcome could be avoided. The sequencing of all 29 *NPHS1* exons did not reveal any homozygous mutations in MCNS patients. However, heterozygous nucleotide changes were common especially in patients with severe form of MCNS, suggesting that genetic factors may play a role in the development and severity of MCNS.

METHODS

Subjects

The study group consisted of 25 adults who had been treated for MCNS in the Hospital for Children and Adolescence, University of Helsinki, during the period of 1965 to 1980. Clinical data including the medication, number of relapses, and laboratory values were recorded. The patients were selected for the *NPHS1* analysis because they all had a biopsy-proven MCNS and a clinical disease of variable severity. A clinical questionnaire was send to all patients to clarify the present health status. The patients' possible signs of renal disease were verified by measuring serum creatinine, albumin, cholesterol, and hemoglobin values, and performing urinalysis. Blood samples were collected for the DNA analyses. A family history was taken, and absence of nephrotic syndrome in the first-degree relatives was required.

The clinical data of the 25 patients are presented in Tables 1 and 3. The severity of the disease varied greatly, and three patients still had nephrotic periods as adults; however, none showed any sign of renal failure. For statistical analyses the patients could be divided in groups according to the classification of the International Study Group [1]. Twenty patients were regarded as steroid-sensitive cases. Nine of them had infrequent relapses, and 11 had frequent relapses (at least four episodes/year). Six of the patients in the latter group also showed steroid dependence. Steroid resistance was observed in five cases. It was defined as a failure to achieve response in spite of four weeks' therapy with prednisone (60 mg/m^2/day). One of these five was an early nonresponder (patient 21 in Table 3), and two were classified as late nonresponders (patients 23 and 25). In the latter two cases remission was achieved with steroids within a week in the first proteinuric episode. However, later episodes showed variable responsiveness to both steroids and alkylating agents. While the duration of the disease was 2.5 years in the first case, the other patient was still having nephrotic periods in adulthood. Two patients (patients 22 and 24) had no clear response to steroid treatment in the initial episode. Both patients attained remission after a course of cytotoxic therapy.

Control patients included 35 young adults with no personal or family history of kidney diseases. Both study subjects and control subjects were of the Finnish origin.

Methods

Genomic DNA was isolated according to standard laboratory protocol from frozen or fresh peripheral blood samples using conventional molecular biology technique (Puregene EPTM DNA Purification Kit; Gentra Systems, Minneapolis, MN, USA). The molecular analysis of the nephrin gene was performed using direct sequencing. Exons were amplified by polymerase chain reaction (PCR) with flanking intron primers designed for exons 1 to 29, and the reactions were performed in total volumes of 25 μL as previously described [11]. Occasionally, the denaturation temperature was raised up to 98°C (exons 12 and 13), or betaine was added to the reaction mixture. PCR products were subjects to automated sequence analysis by bigdye-terminator reactions (Automated Sequencer ABI 377 and Genetic Analyzer 3100; Applied Biosystems, Foster City, CA, USA). All 29 exons were sequenced from the 25 MCN patients. Eleven exons [3, 8, 9, 10, 11, 17–20, 24, 26] showed sequence variants, and were

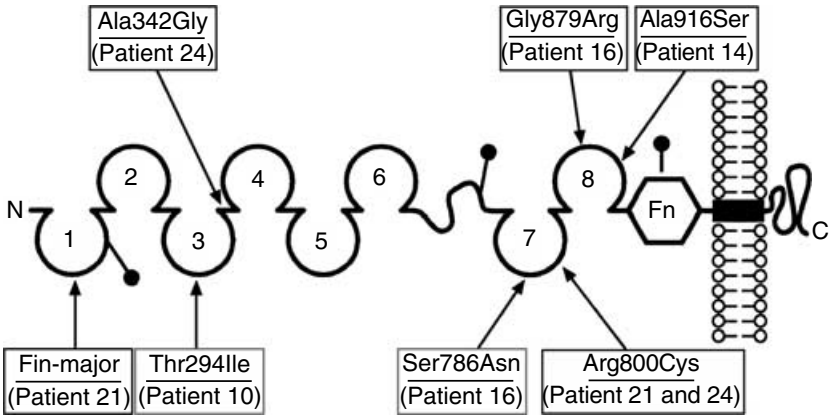

Fig. 1. Amino acid substitutions in five patients with minimal change nephrotic syndrome (MCNS). The changes were found in a spacer area between immunoglobulin (Ig) domain 3 and 4 (A342G), in Ig-7 domain (R800C), and in Ig-8 domain (G879R, A916S). Two changes occur at nonconserved sites in the Ig-3 (T294I) and Ig-7 (S786N).

analyzed from the 25 control subjects. In addition, the five exons [8, 9, 18–20] carrying new amino acid changes were analyzed from 10 additional control subjects so that the total number of control chromosomes was 70.

Statistical analysis was done by Student *t* test. The study was approved by the Ethics Committee of the Hospital for Children and Adolescents, University of Helsinki. Informed consent was obtained from all patients.

RESULTS

Allelic variants of NPHS1

All 29 exons in the *NPHS1* gene were directly sequenced from the DNA of 25 MCNS patients. No homozygous mutations were observed in MCNS patients. While Fin-major mutation in exon 2 was observed in one patient, none of the patients had Fin-minor mutation in exon 26. A total of 12 sequence variants were observed in 11 exons, as summarized in Table 2. To examine the significance of these changes, the same exons from the DNA of 25 control subjects were also sequenced (Table 2), and the results were compared with the published data [11, 12, 19, 20] and public databases (dBSNP, SNPper).

Six of the 12 nucleotide changes were known polymorphic variants (SNP) (Table 2). Three of these caused an amino acid substitution, and three had no effect on the amino acid sequence of nephrin. Nucleotide changes were especially common in exons 3 and 26. The number of variants in the 11 exons tested varied from 1 to 6 (mean 3.0) in MCNS patients, and from 0 to 5 (mean 2.6) in control subjects (P = NS) (Tables 2 and 3). Nine of the 20 steroid-sensitive MCNS patients had a mild disease (1–4 relapses), and 11 were frequent relapsers (4–27 episodes). The mean number of nucleotide changes was 2.3 and 3.5 in the two groups, respectively ($P < 0.05$). Six of the frequent relapsers were steroid-dependent and had, on average, 4.2 nucleotide changes compared with patients with no dependence to steroids (mean 2.4) ($P = 0.001$). The mean number of amino acid substitutions was 2.0 and 1.4 in the two groups, respectively (P = NS).

MCNS patients with nonconservative amino acid substitutions

Five MCNS patients had six heterozygous allelic variants, resulting in amino acid changes of nephrin protein (Table 2, Fig. 1). Five of the six amino acid changes were previously unreported.

Patient no. 16 (Table 3) had a nucleotide change of 2635G>A in exon 19, causing a substitution of neutral glycine by positively charged arginine (G879R) at a conserved site of Ig-8 domain, and a nucleotide change 2357G>A in exon 18, causing a conservative amino acid substitution of serine by asparagine (S786N) at a nonconserved site of Ig-7 domain. The patient had 18 proteinuric episodes in childhood. After 18 years of remission, one relapse occurred in adulthood. The present kidney function of the patient is normal (serum creatinine 89 μmol/L). The mother of this patient carried only the substitution G879R, suggesting that the two variants in this patient were in different NPHS1 alleles. As the father of the patient was deceased, DNA sample was not available for the analysis.

Patient no. 24 had a 2398C>T change in exon 18, leading to a substitution of basic arginine by cysteine (R800C) at a conserved site in the Ig-7 domain, and a 1025C>G transition in exon 9, causing a conservative amino acid substitution of alanine by glycine (A342G) at a conserved site between motifs Ig-3 and Ig-4. This patient responded poorly to steroid treatment. The duration of the disease was 5.8 years, and the present kidney function is good (serum creatinine 86 μmol/L). Both parents of this patient were deceased, which hampered the analysis of the allelic variants. The R800C-substitution was found in a heterozygous form in one of the 35 control subjects.

Patient no. 21 had a Fin-major mutation in exon 2, leading to a truncated nephrin peptide of only 90 amino acids and the same nonconservative R800C substitution in Ig-7 domain as patient 24. The initial proteinuric episode at the age of seven years responded poorly to steroids (early nonresponder), but the four relapses were successfully

Table 2. Amino acid substitutions and polymorphisms of the NPHS1 gene in 25 MCNS patients and 25 or 35 control subjects

Exon	Nucleotide change	Effect on protein	No. of changes per alleles		Comment
			MCNS	Control subjects	
9	1025C→G	A342G	1/50	0/70	New conservative amino acid substitution in patient no. 24. Alanine is substituted by glycine. Both amino acids are neutral and hydrophobic. Change occurs at conserved site in the area between motifs Ig-3 and Ig-4.
18a	2357G→A	S786N	1/50	0/70	New conservative amino acid change (serine→asparagine) in patient no. 16 with continuing nephrotic periods in adulthood. Both amino acids are neutral and contain nonpolar side chain. Change occurs at nonconserved site in the Ig-7 domain.
18b	2398C→T	R800C	2/50	1/70	New non-conservative amino acid substitution was found in patients no 21 and 24, who did not respond to steroid treatment. Hydrophilic and basic arginine is substituted by neutral cysteine containing reactive sulfhydryl group. Change occurs at conserved site in the Ig-7 domain.
19	2635G→A	G879R	1/50	0/70	New nonconservative amino acid substitution in patient no. 16 with continuing nephrotic periods in adulthood. Neutral glycine with nonpolar side chain is substituted by positively charged arginine. Change occurs at conserved site in the Ig-8 domain.
20	2746G→T	A916S	1/50	0/70	New nonconservative amino acid substitution in patient no. 14. Hydrophobic alanine with nonpolar side chain is converted to hydrophilic serine with polar side chain. Change occurs at conserved site in the Ig-8 domain.
8	881C→T	T294I	1/50	0/70	Nonconservative amino acid change from hydrophilic threonine to neutral isoleucine in patient no. 10. Previously found also in a mother of a CNF child, Beltcheva et al, 2001. Change occurs at a site in the Ig-3 domain, which is not conserved during evolution.
3	349 G→A	E117K	24/50	20/50	Known polymorphism. Amino acid change: glutamic acid→lysine (Lenkkeri et al, 1999).
10	1223G→A	R408Q	4/50	3/50	Known polymorphism. Amino acid change: arginine→glutamine (Lenkkeri et al, 1999).
24	3230A→G	N1077S	3/50	9/50	Known polymorphism. Amino acid change: asparagine→serine (Lenkkeri et al, 1999).
11	1320C→T	P440P	2/50	4/50	New polymorphism. No amino acid change.
17	2289C→T	V763V	2/50	4/50	New polymorphism. No amino acid change.
26	3315G→A	S1105S	32/50	24/50	Known polymorphism. No amino acid change (Beltcheva et al, 2001).

The number of nucleotide changes per 50 or 70 alleles. The patient number refers to Table 3.

treated with prednisone. The duration of the disease was 5.1 years. The patient is now 40 years old and is doing clinically well, with a slightly elevated serum creatinine value (126 μmol/L). The analysis of DNA sample from the mother of this patient revealed that he carried only the nucleotide change R800C, suggesting that the two variants in the patient were in different alleles. Because the father of this patient was deceased and no DNA sample was available, a de novo mutation (Fin-major) cannot totally be excluded. However, based on the experience on the CNF families, a sporadic Fin-major mutation in this patient seems very unlikely.

Patient no. 10 had a nucleotide change of 881C>T in exon 8, causing a substitution of hydrophilic threonine by neutral isoleucine (T294I) at a nonconserved site in Ig-3 domain, and a nucleotide change of 3230A>G in exon 24, leading to a known polymorphic amino acid substitution of asparagine by serine (N1077S) in transmembrane part of nephrin polypeptide. Interestingly, this patient had transient proteinuria in infancy before MCNS started at the age of 5 years (Table 3). The T294I substitution has previously been reported in a mother of a CNF child and has been regarded as a polymorphic change [12].

Patient no. 14 had a single change of 2746G-T in exon 20, resulting in a substitution of hydrophobic alanine by hydrophilic serine (A916S) at a conserved site in Ig-8 domain. The patient had 13 relapses in childhood and is now clinically well (Table 3).

DISCUSSION

In this work we studied the possible role of the major slit diaphragm protein, nephrin, in the pathogenesis of MCNS by analyzing the nephrin gene (*NPHS1*) structure in patients with a biopsy-proven MCNS in childhood. While the analysis did not reveal any specific MCNS-associated mutation, five of the 25 patients had heterozygous amino acid substitutions, probably leading to unfavorable conformational changes in nephrin structure. This suggests that genetic defects in glomerular filter proteins may play a role in some patients with MCNS.

Despite careful work in many laboratories, the pathogenesis of MCNS has remained a mystery for decades. While it is believed that the disease has an immunologic basis, no antibody or T-cell–mediated process has been detected in the MCNS kidneys [3, 21]. Alterations in

Table 3. Clinical data, NPHS1 exons, nucleotide, and amino acid changes of the 25 patients with MCNS

							Nucleotide change in NPHS1 exon and effect on protein													
Pt.	Sex	Age at onset *years*	Relapse no.	Steroid sensitive/ resistant	Steroid dependent	Duration of disease *years*	Ex3 nt 349 G→A E117K	Ex8 nt 881 C→T T294I	Ex9 nt 1025 C→G A342G	Ex10 nt 1223 G→A R408Q	Ex11 nt 1320 C→T P440P	Ex17 nt 2289 C→T V763V	Ex18a nt 2357 G→A S786N	Ex18b nt 2398 C→T R800C	Ex19 nt 2635 G→A G879R	Ex20 nt 2746 G→T A916S	Ex24 nt 3230 A→G N1077S	Ex26 nt 3315 G→A S1105S	Total number of SNC	Total number of AC
1	F	5.9	2	S	No	3.6	–	–	–	h	–	–	–	–	–	–	–	H	3	1
2	M	3.3	2	S	No	0.8	h	–	–	–	–	–	–	–	–	–	–	h	2	1
3	F	1.9	1	S	No	0.3	h	–	–	–	–	–	–	–	–	–	–	h	2	1
4	F	2.8	4	S	No	2.8	H	–	–	–	–	–	–	–	–	–	–	–	2	2
5	M	1.6	4	S	No	2.5	h	–	–	–	–	–	–	–	–	–	–	H	3	1
6	M	6.3	4	S	No	5.1	h	–	–	–	–	–	–	–	–	–	h	h	3	2
7	M	6.0	3	S	No	0.7	h	–	–	–	–	–	–	–	–	–	–	h	2	1
8	F	7.0	1	S	No	0.5	H	–	–	–	–	–	–	–	–	–	–	h	3	2
9	F	2.9	3	S	No	4.1	–	–	–	–	–	–	–	–	–	–	–	h	1	0
10	M	5.3	4	S	No	1.0	–	h	–	–	–	–	–	–	–	–	h	h	3	2
11	M	4.0	12	S	No	5.8	H	–	–	–	–	–	–	–	–	–	–	–	2	2
12	M	8.9	13	S	No	4.0	h	–	–	h	–	–	–	–	–	–	–	H	4	2
13	M	2.6	16	S	No	13.0	h	–	–	–	–	–	–	–	–	–	–	h	2	1
14	F	4.9	13	S	No	4.1	–	–	–	–	–	–	–	–	–	h	–	h	2	1
15	M	0.8	25	S	Yes	27.0→	–	–	–	h	h	h	–	–	–	–	–	h	4	1
16	M	5.6	18	S	Yes	25.0→	H	–	–	–	–	–	h	–	h	–	–	H	6	4
17	F	5.5	7	S	Yes	2.9	h	–	–	–	h	h	–	–	–	–	h	h	5	2
18	F	4.8	5	S	Yes	0.9	h	–		–	–	–	–	–	–	–	–	h	2	1
19	M	3.5	27	S	Yes	12.3	H	–	–	–	–	–	–	–	–	–	–	H	5	3
20	M	4.0	19	S	Yes	7.7	H	–	–	–	–	–	–	–	–	–	–	H	4	2
21	M	7.8	4	R	No	5.1	–	–	–	–	–	–	–	h	–	–	–	H	3	1
22	M	3.2	0	R	No	0.5	h	–	–	–	–	–	–	–	–	–	–	–	1	1
23	M	6.4	10	R	No	2.5	H	–	–	–	–	–	–	–	–	–	–	H	4	2
24	F	14.7	0	R	No	5.8	–	–	h	h	–	–	–	h	–	–	–	H	5	3
25	M	2.7	28	R	No	27→	–	–	–	–	–	–	–	–	–	–	–	H	2	0

H, homozygous change; h, heterozygous change. Patients 1–9 had a mild disease (1–4 relapses), patients 10–20 had frequent relapses, and patients 21–25 showed steroid resistance. Patients in bold are described in detail in the **Results**. Patient 21 also carried a Fin-major mutation.

the T-lymphocyte subpopulations in the peripheral blood during relapses have been reported. Also, changes in the cytokine production have been found in MCNS patients [22]. As is the case with focal segmental glomerulosclerosis (FSGS), the production of a soluble "proteinuric factor" has been suggested. This factor would penetrate into the glomerular capillary wall and interfere with the filtration barrier.

What goes wrong in the glomerular filter in MCNS is not known. Structural changes in the GBM, such as a decrease in anionic proteoglycans, have been suggested [23]. In animal models, detachment of podocyte foot processes from the GBM have correlated with the onset of proteinuria, suggesting that areas of denuded GBM might serve as the sites of protein leakage [24]. The latest discoveries in the genetic diseases, however, indicate that podocytes and the slit diaphragm are crucial for glomerular ultrafiltration [4–6]. Defects in podocyte proteins, such as nephrin, Neph1, podocin, CD2-associated protein (CD2AP), and alpha-actinin-4, all cause heavy proteinuria [25–28]. Thus, a defective podocyte filtration slit is a probable route for protein leakage.

Nephrin is an important component of the glomerular filter. Absence of nephrin leads to a distortion of the slit diaphragm, which can be seen in the Finnish CNF patients, who have no slit diaphragm filaments in the electron microscopy [10]. A similar phenomenon was recently seen also in MCNS kidneys [14]. Also, altered expression of nephrin, as studied by light and electron microscopy, has been reported in MCNS [15, 16, 29]. Based on these data we hypothesized that MCNS patients might have a genetically vulnerable glomerular filter. Because of its central role, a defective nephrin would easily predispose to proteinuria.

Our analysis revealed considerable frequency of allelic NPHS1 variants in the 100 chromosomes from MCNS patients and control subjects. A total of 12 nucleotide changes were observed in 11 exons. Six of the 12 nucleotide changes were known polymorphisms (SNP). Three of these caused an amino acid substitution, and three had no effect on the amino acid sequence of nephrin. Previously, eight exonic SNPs and about 40 intronic SNPs have been registered in public databases (dBSNP and SNPper). Five of the exonic SNPs (E117K, P440P, V763V, N1077S, S1105S) were found also in our patients. Three reported polymorphisms (T741T, S440S, and I98I) were not found either in our patients or in the control subjects. The average number of sequence variants was not significantly higher in MCNS patients compared with control subjects (3.0 vs. 2.6). Within MCNS, there was a tendency for a higher number of variants in patients with a complicated disease (steroid dependency) as compared with those with a mild disease (4.2 and 2.4 variants, respectively). Because this difference was mainly based on nucleotide changes not affecting nephrin structure, the significance of the finding remains open.

The frequency of heterozygous Fin-major and Fin-minor carriers in the Finnish population is high (about 2%), and it was interesting to see whether the carriership would predispose to MCNS [30]. This was not the case, because only one of the 25 MCNS patients had heterozygous Fin-major mutation. Heterozygous carriers of Fin-major or Fin-minor have only one functional allele, which is normally enough (e.g., parents of the CNF children do not have proteinuria and can be used as donors in kidney transplantation). However, during the fetal period, *NPHS1* mutation carriers have temporary proteinuria, indicating the necessity of both alleles for sufficient synthesis of nephrin during glomerulogenesis [31]. Thus, it seems that after glomerulogenesis is finished, one normal allele is enough even in a "stress condition" such as MCNS.

One MCNS patient in this analysis had the Fin-major mutation in one *NPHS1* allele, and a 2398C>T nucleotide change in the other, causing a substitution of arginine by cysteine in Ig-7 domain of nephrin (R800C). MCNS manifested in this patient at the age of 7 years and lasted for 5 years. It is interesting that six Finnish CNF patients had a similar situation, with the Fin-major mutation in one allele and a missense mutation in the other. Five of them have received kidney transplantation. However, one patient has the substitution of arginine by cysteine in the spacer area close to Ig7-motif (R743C). The patient had full-blown nephrotic syndrome immediately after birth, but responded to the anti-proteinuric therapy with indomethacin and ACE-inhibitor and, as yet, is the only Finnish CNF patient (at the age of 5 years) who has not needed kidney transplantation [10]. Thus, the Finnish patients with compound Fin-major and missense changes show a graded severity of disease from CNF to MCNS. This variation may be explained by the type of the amino acid substitution that takes place in the nephrin polypeptide. Liu et al [32] have shown that most missense mutations associated with typical CNF lead to a defective folding of the synthesized nephrin, so that the molecule never reaches the podocyte surface. A less severe change (as was the case with the R743C substitution) would lead to a nephrin molecule that is expressed at the slit diaphragm but is functionally defective and may cause "atypical" phenotype. We analyzed 35 healthy control subjects, and the R800C substitution was found in a heterozygous form in only one of them. Both of our patients carrying R800C (patients 21 and 24) also had another defect in their nephrin gene. Therefore, R800C is most likely a pathogenic alteration and affected the phenotype of patient 21, who had Fin-major mutation in the other allele. Four additional MCNS patients had heterozygous, nonconservative amino acid substitutions in nephrin (G879R; R800C; T294I; A916S). Three of these were at conserved

sites in the Ig-domains based on the comparison of human, rat, and mouse sequences. Two of the patients also had additional conservative amino acid substitutions (S786N; A342G). The variants were not found in public databases dBSNP or SNPper, suggesting that they are not common polymorphic alterations. The pathogenic role of the amino acid substitutions remains to be verified, but it seems possible that they have a dominant-negative effect on the slit diaphragm architecture. The situation would then be analogous to that of the other glomerular filter, the GBM, where heterozygous mutations in type IV collagen alpha 3 and 4 genes (*COL4A3, COL4A4*) can have a dominant-negative effect on the collagen network and the GBM structure, resulting in a continuous spectrum of phenotypes from classic Alport syndrome to benign familial hematuria [33, 34]. Nephrin interacts with other slit diaphragm components, such as Neph1, podocin, and CD2AP, and also takes part in cell signaling [25, 27, 35–37]. It seems possible that amino acid changes in critical points of the protein would interfere with these functions. The change of arginine by cysteine (R800C) in Ig-7 domain probably causes formation of incorrect disulfide bonds, but otherwise, the impact of each amino acid substitution on the protein structure cannot, so far, be predicted because the crystal molecular structure of nephrin is not yet known. It is, however, interesting that four of the amino acid substitutions in our MCNS patients occurred in Ig-7 and Ig-8 domains, which show clustering of mutations also in CNF patients [12, 13].

Nephrin is not the only slit diaphragm component, and it is clear that genetic variation in other podocyte proteins, such as podocin, NEPH1, CD2AP, and α-actinin-4 may equally affect the filter function. Podocin is especially interesting in this respect. Mutations in podocin gene (*NPHS2*) cause the autosomal-recessive form of FSGS, as well as some of the "sporadic" FSGS cases [26, 38, 39]. Some adult patients with FSGS have been found to bear a heterozygous mutation (R229Q) that enhances susceptibility to FSGS in association with a second mutant *NPHS2* allele [40]. Interestingly, podocin with the R229Q amino acid change bound poorly to nephrin, underscoring the importance of the interplay with these two proteins. Furthermore, Kim et al [41] found recently that mice that had CD2AP haploinsufficiency showed increased susceptibility to glomerular injury by nephrotoxic antibodies and immune complexes. They also identified two human patients who were heterozygous for CD2AP mutations and still had proteinuria, implicating CD2AP as a determinant of susceptibility to a glomerular disease. It is also possible that mutations in two or more genes are needed to make the glomerular filter susceptible to a pathogenic process. The collaborative effect of the NPHS1 and NPHS2 genes has already been shown. Koziel et al reported an overlap in the NPHS1/NPHS2 mutations, and characterized a di-genic inheritance, resulting in a "tri allelic" hit, which converted the phenotype from CNF to the congenital form of FSGS [13].

CONCLUSION

MCNS is thought to be a multifactorial disease, and the basic mechanisms behind MCNS still wait to be solved. The results here, however, suggest for the first time that genetic variants of a glomerular filter protein may play a role in MCNS. Expanding the genetic work to the other podocyte proteins therefore seems indicated.

ACKNOWLEDGMENTS

We thank Maija Suvanto, Taija Havulinna, and Tuija Heinonen for excellent technical assistance. This work was supported by grants from the Finnish Academy, the Sigrid Juselius Foundation, Pediatric Research Foundation, Helsinki University Central Hospital Research Fund, and Research Foundation of Orion Corporation.

Reprint requests to Hannu Jalanko, Hospital for Children and Adolescents, University of Helsinki, 00290 Helsinki, Finland.
E-mail: hannu.jalanko@hus.fi

REFERENCES

1. Clark AG, Barratt TM: Steroid-responsive nephrotic syndrome, in *Pediatric Nephrology* (vol 4), edited by Barratt TM, Avner ED, Harmon WE, Baltimore, Lippincott Williams and Wilkins, 1999, pp 731–747
2. Koskimies O, Vilska J, Rapola J, Hallman N: Long-term outcome of primary nephrotic syndrome. *Arch Dis Child* 57:544–548, 1982
3. Grimbert P, Audard V, Remy P, *et al*: Recent approaches to the pathogenesis of minimal-change nephrotic syndrome. *Nephrol Dial Transplant* 18:245–248, 2003
4. Tryggvason K: Unraveling the mechanisms of glomerular ultrafiltration: Nephrin, a key component of the slit diaphragm. *J Am Soc Nephrol* 10:2440–2445, 1999
5. Jalanko H, Patrakka J, Tryggvason K, Holmberg C: Genetic kidney diseases disclose the pathogenesis of proteinuria. *Ann Med* 33:526–533, 2001
6. Mundel P, Shankland SJ: Podocyte biology and response to injury. *J Am Soc Nephrol* 13:3005–3015, 2002
7. Kestilä M, Lenkkeri U, Männikkö M, *et al*: Positionally cloned gene for a novel glomerular protein—nephrin—is mutated in congenital nephrotic syndrome. *Mol Cell* 1:575–582, 1998
8. Holmberg C, Jalanko H, Tryggvason K, Rapola J: Congenital nephrotic syndrome, in *Pediatric Nephrology*, edited by Barratt T, Avner E, Harmon W, Baltimore, Lippincott Williams & Wilkins, 1999, pp 765–778
9. Huttunen NP: Congenital nephrotic syndrome of the Finnish type. Study of 75 patients. *Arch Dis Child* 51:344–348, 1976
10. Patrakka J, Kestilä M, Wartiovaara J, *et al*: Congenital nephrotic syndrome (NPHS1): Features resulting from different mutations in Finnish patients. *Kidney Int* 58:972–980, 2000
11. Lenkkeri U, Männikkö M, McCready P, *et al*: Structure of the gene for congenital nephrotic syndrome of the finnish type (NPHS1) and characterization of mutations. *Am J Hum Genet* 64:51–61, 1999
12. Beltcheva O, Martin P, Lenkkeri U, Tryggvason K: Mutation spectrum in the nephrin gene (NPHS1) in congenital nephrotic syndrome. *Hum Mutat* 17:368–373, 2001
13. Koziell A, Grech V, Hussain S, *et al*: Genotype/phenotype correlations of NPHS1 and NPHS2 mutations in nephrotic syndrome advocate a functional inter-relationship in glomerular filtration. *Hum Mol Genet* 11:379–388, 2002
14. Patrakka J, Lahdenkari AT, Koskimies O, *et al*: The number of podocyte slit diaphragms is decreased in minimal change nephrotic syndrome. *Pediatr Res* 52:349–355, 2002

15. Furness PN, Hall LL, Shaw JA, Pringle JH: Glomerular expression of nephrin is decreased in acquired human nephrotic syndrome. *Nephrol Dial Transplant* 14:1234–1237, 1999
16. Wernerson A, Duner F, Pettersson E, *et al*: Altered ultrastructural distribution of nephrin in minimal change nephrotic syndrome. *Nephrol Dial Transplant* 18:70–76, 2003
17. Patrakka J, Ruotsalainen V, Ketola I, *et al*: Expression of nephrin in pediatric kidney diseases. *J Am Soc Nephrol* 12:289–296, 2001
18. Kim BK, Hong HK, Kim JH, Lee HS: Differential expression of nephrin in acquired human proteinuric diseases. *Am J Kidney Dis* 40:964–973, 2002
19. Aya K, Tanaka H, Seino Y: Novel mutation in the nephrin gene of a Japanese patient with congenital nephrotic syndrome of the Finnish type. *Kidney Int* 57:401–404, 2000
20. Bolk S, Puffenberger EG, Hudson J, *et al*: Elevated frequency and allelic heterogeneity of congenital nephrotic syndrome, Finnish type, in the old order Mennonites. *Am J Hum Genet* 65:1785–1790, 1999
21. Cunard R, Kelly CJ: T cells and minimal change disease. *J Am Soc Nephrol* 13:1409–1411, 2002
22. Lama G, Luongo I, Tirino G, *et al*: T-lymphocyte populations and cytokines in childhood nephrotic syndrome. *Am J Kidney Dis* 39:958–965, 2002
23. Raats CJI, Van Den Born J, Berden JHM: Glomerular heparan sulfate alterations: Mechanism and relevance for proteinuria. *Kidney Int* 57:385–400, 2000
24. Messina A, Davies DJ, Dillane PC, Ryan GB: Glomerular epithelial abnormalities associated with the onset of proteinuria in aminonucleoside nephrosis. *Am J Pathol* 126:220–229, 1987
25. Sellin L, Huber TB, Gerke P, *et al*: NEPH1 defines a novel family of podocin interactin proteins. *FASEB J* 17:115–117, 2003
26. Boute N, Gribouval O, Roselli S, *et al*: NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* 24:349–354, 2000
27. Shih NY, Li J, Karpitskii V, *et al*: Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* 286:312–315, 1999
28. Kaplan JM, Kim SH, North KN, *et al*: Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nature Genet* 24:251–256, 2000
29. Doublier S, Ruotsalainen V, Salvidio G, *et al*: Nephrin redistribution on podocytes is a potential mechanism for proteinuria in patients with primary acquired nephrotic syndrome. *Am J Pathol* 158:1723–1731, 2001
30. Pastinen T, Perola M, Ignatius J, *et al*: Dissecting a population genome for targeted screening of disease mutations. *Hum Mol Genet* 10:2961–2972, 2001
31. Patrakka J, Martin P, Salonen R, *et al*: Proteinuria and prenatal diagnosis of congenital nephrosis in fetal carriers of nephrin gene mutations. *Lancet* 359:1575–1577, 2002
32. Liu L, Done SC, Khoshnoodi J, *et al*: Defective nephrin trafficking caused by missense mutations in the NPHS1 gene: Insight in to the mechanisms of congenital nephrotic syndrome. *Hum Mol Genet* 10:2637–2644, 2001
33. Longo I, Porcedda P, Mari F, *et al*: COL4A3/COL4A4 mutations: From familial hematuria to autosomal-dominant or recessive Alport syndrome. *Kidney Int* 61:1947–1956, 2002
34. Buzza M, Dagher H, Wan YY, *et al*: Mutations in the COL4A4 gene in thin basement membrane disease. *Kidney Int* 63:447–453, 2003
35. Huber TB, Kottgen M, Schilling B, *et al*: Interaction with podocin facilitates nephrin signaling. *J Biol Chem* 276:41543–41546, 2001
36. Schwarz K, Simons M, Reiser J, *et al*: Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. *J Clin Invest* 108:1621–1629, 2001
37. Roselli S, Gribouval O, Boute N, *et al*: Podocin localizes in the kidney to the slit diaphragm area. *Am J Pathol* 160:3–5, 2002
38. Frishberg Y, Rinat C, Megged O, *et al*: Mutations in NPHS2 encoding podocin are a prevalent cause of steroid-resistant nephrotic syndrome among Israeli-Arab children. *J Am Soc Nephrol* 13:400–405, 2002
39. Karle SM, Uetz B, Ronner V, *et al*: Novel mutations in NPHS2 detected in both familial and sporadic steroid-resistant nephrotic syndrome. *J Am Soc Nephrol* 13:577–579, 2002
40. Tsukaguchi H, Sudhakar A, Le TC, *et al*: NPHS2 mutations in late-onset focal segmental glomerulosclerosis: R229Q is a common disease-associated allele. *J Clin Invest* 110:1659–1666, 2002
41. Kim JM, Wu H, Green G, *et al*: CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility. *Science* 300:1298–1300, 2003